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Influence of KF, DCMU and removal of Ca^{2+} on the high-spin EPR signal of the cytochrome *b*-559 heme Fe(III) ligated by OH^- in chloroplasts

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Abstract

An EPR signal at $g = 6.8$ attributed to the cytochrome (Cyt) *b*-559 heme Fe(III) ligated by OH^- (Fiege, R., Schrieber, U., Lubitz, W., Renger, G. and Shuvalov, V.A. (1995) FEBS Lett. 377, 325–329) was studied. This signal is observed in intact chloroplasts when oxidized by 10 mM 2,3-dicyano,5,6-dichloro-*p*-benzoquinone (DDQ), but not when 5 mM *p*-benzoquinone is added. Addition of KF (100 mM) or removal of Ca^{2+} for blocking the water-oxidizing complex considerably decreases the heme Fe(III)-OH^- EPR signal. In contrast, DCMU does not decrease this signal and does not influence its photochemical changes at 140 K. Thus, the EPR spectrum of Cyt *b*-559 Fe(III) ligated by OH^- is not affected by changes at the acceptor side of Photosystem II, and its photochemical decrease is probably not due to reduction via the acceptor side. Comparison of the effect of KF on the model heme Fe(III) in myoglobin (Mb) at pH 10.5 shows that F^- replaces OH^- as a ligand at the sixth coordination position of the heme Fe(III) in both Mb and chloroplasts Cyt *b*-559. This replacement is accompanied by changes of the symmetry of the molecular field causing a disappearance of the EPR signals at $g = 6.8$ and 5.0. Our results provide further evidence for a possible participation of the Cyt *b*-559 heme Fe ligated by OH^- in photosynthetic water oxidation.

Keywords: Photosystem II; Cytochrome *b*-559; Water oxidation

1. Introduction

The cytochrome (Cyt) *b*-559 protein is a constituent of the reaction center complex of Photosystem II [1]. Optical and EPR studies revealed several forms of Cyt *b*-559 in D1D2Cyt *b*-559 complexes with different redox potentials at different pH [2,3]. The optical and EPR properties of one form at pH 9.4 clearly show that its heme is ligated by OH^- at the sixth coordination position [3].

In intact chloroplasts the major fraction of Cyt *b*-559 is present in a high-potential form ($E_m = +380$ mV) [4]. It was found that oxidation of intact chloroplasts by DDQ [5] leads to the appearance of an EPR signal mostly in the region of the high-spin heme Fe(III). A signal at $g = 6.8$ was attributed to the Cyt

Abbreviations: BQ, *p*-benzoquinone; Cyt *b*-559, cytochrome *b*-559; DDQ, 2,3-dicyano,5,6-dichloro-*p*-benzoquinone; Mb, myoglobin

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b-559 heme Fe(III) ligated by OH^- at the sixth coordination position. This type of ligation comprised a considerable part ($\approx 40\%$) of the total Cyt *b*-559 content. Removal of the Mn-cluster connected to the water-oxidizing complex caused a change of the EPR parameters of the OH^--ligated heme. In intact chloroplasts oxidized by DDQ the EPR signal of the heme Fe(III) ligated by OH^- considerably decreased after illumination at 80 K and 140 K by red light [5]. This data was discussed in relation to the possible function of Cyt *b*-559 in photosynthetic water oxidation.

In the present work, the effect of addition of F^--ions and removal of Ca^{2+}, treatments which inhibit the water-oxidizing complex, on the ligation of Cyt *b*-559 heme Fe(III) by OH^- was studied. It was found that both treatments considerably decrease the EPR signal of the heme Fe(III)-OH^- complex. In contrast, DCMU does not decrease this signal, nor does it influence its photochemical change at 140 K. These results provide further evidence for a possible participation of the Cyt *b*-559 heme Fe ligated by OH^- in photosynthetic water oxidation.

2. Materials and methods

Intact chloroplasts were isolated from spinach leaves as described earlier [6]. Approximately 400 g of leaves were homogenized briefly in a medium consisting of 300 mM sorbitol, 30 mM KCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4, 50 mM MES (pH 6.1) (buffer A). Coarse filtration of the homogenate was followed by centrifugation at $4000 \times g$ for 4 min (4°C) and purification on a Percoll gradient in a buffer identical to A, except that MES was replaced by 50 mM Hepes (pH 7.5) (buffer B). The intactness of the chloroplasts was approximately 90% as determined by measurements of the oxygen evolution rate in the absence and in the presence of $K_3[Fe(CN)_6]$.

Extraction of Ca^{2+} from chloroplasts was done as described [7]. The chloroplasts were resuspended in 1 M NaCl, 400 mM sucrose and 40 mM MES-NaOH (pH 6.5) to a chlorophyll concentration of 0.5 mg/ml, and incubated under ambient light at 4°C for 30 min. The chloroplasts were then washed with 400 mM sucrose, 20 mM NaCl, and 40 mM Hepes-NaOH (pH 7.5), and resuspended in the same medium.

The chlorophyll concentration of the chloroplasts used for the EPR experiments was 10–14 mg/ml. The myoglobin concentration was 0.5 mM in 50 mM Tris buffer (pH 10.5). Chloroplasts in corresponding buffers and Mb in Tris buffer were first frozen to 77 K and then frozen to 10 K for the measurements. The EPR spectra were recorded at 10 K using a Varian spectrometer equipped with an Oxford ESR-9 helium flow cryostat. The samples were illuminated in the cavity at 140 K by red light for 15 min where indicated.

3. Results and discussion

Fig. 1A shows the EPR spectra in the high-spin region of the heme Fe(III) of Mb at pH 10.5 (solid line) and in the presence of 100 mM KF (dotted line). The solid curve shows the spectrum characteristic for

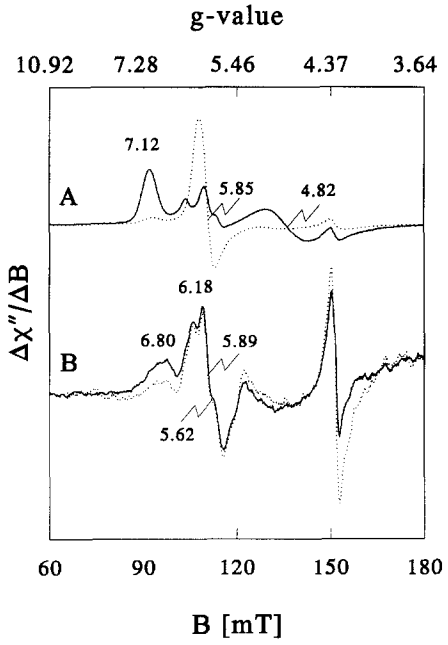

Fig. 1. EPR spectra and their differences in the high-spin region of the heme Fe(III) of intact chloroplasts and Mb. (A) EPR spectrum of 0.5 mM Mb at pH 10.5 (solid line) and in the presence of 100 mM KF (dotted line). (B) Difference EPR spectrum of intact chloroplasts oxidized by 10 mM DDQ with chloroplasts oxidized by 5 mM BQ (solid line). The dotted line shows the light–dark EPR spectrum of DDQ oxidized chloroplasts. Illumination for 15 min at 140 K. Experimental conditions: microwave power 5 mW, modulation amplitude 1 mT, recording time 30 min, temperature 10 K.

the heme Fe(III) ligated by OH^- at high pH (see [5]) with low- and high-field splitting components at g = 7.12 and 4.82, corresponding to octahedral molecular field symmetry with rhombic and tetragonal distortion [8]. The addition of KF leads to a considerable increase of the central line at $g = 5.85$, corresponding to octahedral symmetry with tetragonal distortion [8]. This is due to the replacement of OH^- by F^-, which is a stronger ligand for the heme Fe(III) [9]. The ratio of amplitudes multiplied by the widths of the signals at $g = 7.12$ in the absence of KF and at $g = 5.85$ in the presence of KF is 1:1.67 (a little different from the ratio (1:1.25) found for Mb at pH 7.5 and 10.5 [5]).

Fig. 1B (solid line) shows the difference EPR spectrum of the high-spin region of the heme Fe(III) for intact chloroplasts oxidized by 10 mM DDQ ($E_m \approx 500$ mV at pH 7 [10]) and by 5 mM BQ ($E_m \approx 290$ mV at pH 7). One sees that the additional oxidation by DDQ causes the appearance of a signal at $g = 5.89$ with two low-field splitting components having a sharp shoulder at $g = 6.18$ and a broad shoulder at 6.86. A high-field splitting component with $g = 5.62$ is clearly seen. A second component at $g \approx 5.0$, superimposed on a broad through (see below), can also be observed. Illumination of the sample oxidized by DDQ with red light ($\lambda > 600$ nm) at 140 K for 15 min leads to a considerable decrease of the $g = 6.8$ component (dotted line), in agreement with previous data [5].

The $g = 6.8$ component is assigned to the heme Fe(III) of Cyt *b*-559 ligated by OH^- (see ref. [5]) on the basis of a comparison with EPR spectra of the heme Fe(III) in Mb (Fig. 1A). The ratio of amplitudes multiplied by the widths of the signals at $g = 6.8$ and at $g = 5.8$ (including $g = 6.2$) is 1:1.73, respectively, which is very close to the ratio found for Mb (see above). This shows that about 50% of the high-spin heme Fe(III) is ligated by OH^-.

Fig. 2A (solid line) shows the difference EPR spectrum of chloroplasts oxidized by 10 mM DDQ in the presence of 100 μM DCMU and chloroplasts oxidized by 5 mM BQ. This difference spectrum is very similar to that obtained in the absence of DCMU. The dotted curve shows the difference (light–dark) spectrum for the sample oxidized by DDQ in the presence of DCMU and then illuminated for 15 min at 140 K. In agreement with data obtained for chloroplasts in the absence of DCMU (ref. [5] and Fig. 1B), a decrease of the $g = 6.8$ signal is observed, accompanied by an increase of the signals at $g = 5.8$ and at $g = 6.2$. These results show that DCMU does not alter the heme Fe(III) ligated by OH^-. Light-induced changes are also not effected by DCMU. Since DCMU influences the acceptor side, these changes are probably not due to a reduction of the heme Fe(III) via this side of the reaction center. Therefore, the photo-induced decrease of the $g = 6.8$ signal is rather related to photo-oxidation of the heme Fe(III)-OH^-–Mn-cluster complex, or to removal of OH^- from the heme Fe(III) with formation of the heme Fe(III) lacking the sixth ligand, as suggested earlier [5].

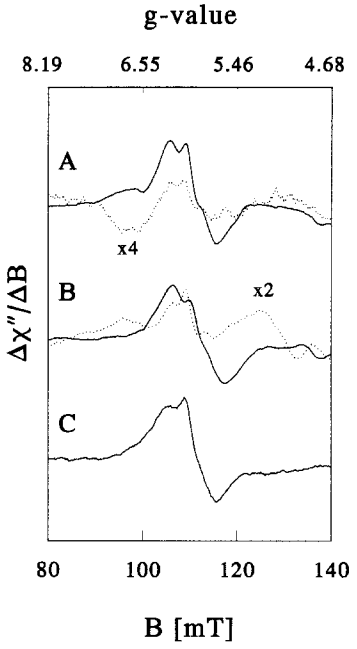

Fig. 2. Difference EPR spectra in the high-spin region of the heme Fe(III) of intact chloroplasts. (A) Difference spectrum of chloroplasts oxidized by 10 mM DDQ in the presence of 100 μM DCMU and chloroplasts oxidized by 5 mM BQ (solid line). The dotted curve (increased by a factor of 4) shows the difference (light–dark) spectrum for the sample oxidized by DDQ and illuminated for 15 min at 140 K. (B) Difference spectrum of chloroplasts in the presence 100 mM KF with and without DDQ (solid line). The dotted curve (increased by a factor of 2) shows the difference spectrum of chloroplasts oxidized by DDQ in the absence and presence of 100 mM KF. (C) Difference spectrum of Ca^{2+}-depleted chloroplasts with and without DDQ. Experimental conditions: microwave power 5 mW, modulation amplitude 1 mT, recording time 30 min, temperature 10 K.

Fig. 2B (solid line) shows that in the presence of 100 mM KF the EPR spectrum of the heme Fe(III) of Cyt *b*-559 is changed, with almost complete disappearance of the splitting components related to the heme Fe(III)-OH^- at $g = 6.86$ and 5.07. The latter component is clearly seen in the difference EPR spectrum (dotted line) obtained for samples without KF and with KF. From these data and data obtained for Mb (Fig. 1A) one sees that F^- replaces OH^- as a ligand of the heme Fe(III), thereby changing the molecular field symmetry (disappearance of rhombic distortion [8]).

Fig. 2C shows the difference EPR spectrum for control NaCl-washed chloroplasts and for a sample oxidized by 10 mM DDQ. This difference spectrum shows that the low-field splitting component at $g = 6.8$ is not observed while the central line at $g = 5.89$ is increased by oxidation with DDQ. Adding Ca^{2+} to the sample partially restores the line at $g = 6.8$ with the appearance of a broad component (not shown). The restoration is accompanied by the appearance of light-induced changes in this line. These data show that the removal of Ca^{2+} accompanied by loss of oxygen evolution also leads to the loss of the heme Fe(III) ligated by OH^-. This effect is partially reversible by adding Ca^{2+}.

The results shown here support previous conclusions [5] that the heme Fe(III) ligated by OH^- has a high redox potential (between 300 and 500 mV). This potential agrees with that of the high-potential form of Cyt *b*-559 as determined optically [4]. The ligation of OH^- is very sensitive to procedures that inhibit the water-oxidizing complex. However, since all measurements in this work were done with oxidized Cyt *b*-559, there is no information on the ligands of Cyt *b*-559 in the reduced state. It has been shown that removal of the Mn cluster from chloroplasts or the addition of NH_4Cl to chloroplasts for blocking oxygen evolution, changes the heme Fe(III)-OH^- signal at $g = 6.8$ [5]. The addition of KF and removal of Ca^{2+} (probably together with the 16 and 24 kDa proteins [11]), treatments which inhibit the water-oxidizing complex, considerably decrease the EPR signal of heme Fe(III) ligated by OH^-. The low-temperature (at 140 K) light-induced decrease of this signal for oxidized chloroplasts shows that the signal is associated with Cyt *b*-559, which is close to the reaction center of Photosystem II and can be photo-oxidized at low temperature [4], and not with other cytochromes (which cannot be photo-oxidized at low temperature). The absence of an effect of DCMU on the light-induced changes of the signal at $g = 6.8$ shows that these changes are not due to the reduction of the heme Fe(III) via the acceptor side of the reaction center. They are rather due to oxidation of the complex of the heme Fe(III)-OH^-–Mn-cluster, or to removal of OH^- from the heme Fe(III) upon illumination by red light, in agreement with a previous suggestion [5]. A more detailed analysis of the water-oxidizing complex including Cyt *b*-559 is in progress.

Acknowledgements

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References

[1] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[2] Shuvalov, V.A., Schreiber, U. and Heber, U. (1994) FEBS Lett. 337, 226–230.
[3] Shuvalov, V.A., Fiege, R., Schreiber, U., Lendzian, F. and Lubitz, W. (1995) Biochim. Biophys. Acta 1228, 175–180.
[4] Cramer, W.A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, 133–172.
[5] Fiege, R., Schreiber, U., Renger, G., Lubitz, W. and Shuvalov, V.A. (1995) FEBS Lett. 377, 325–329.
[6] Asada, K., Neubauer, C., Heber, U. and Schreiber, U. (1990) Plant Cell Physiol. 31, 557–564.
[7] Ono, T.-A. and Inoue, Y. (1990) Biochim. Biophys. Acta 1020, 269–277.
[8] Wertz, J.E. and Bolton, J.R. (1972) Electron Spin Resonance, McGraw-Hill, New York.
[9] Falk, J.E. (1964) Porphyrins and Metalloporphyrins, Elsevier, Amsterdam.
[10] Bergström, J. and Vänngård, T. (1982) Biochim. Biophys. Acta 682, 452–456.
[11] Debus, R.J. (1992) Biochim. Biophys. Acta 1102, 262–352.